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Pharmacognosy
Phytochemical screening and biological assays of ethanolic leaf extract of *Senna rugosa*

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Abstract
Senna rugosa (Fabaceae) is a common specie of the Brazilian territory, especially in the Cerrado biome. It is widely used in the popular medicine although not yet adequately investigated as to its phytoconstituents and pharmacological activities. In this study, the preliminary phytochemical analysis of ethanolic leaf extract of *S. rugosa* was performed and its cytotoxicity, antitumoral and antimicrobial activities (antibacterial, antifungal, anti-*Trypanosoma* and anti-*Leishmania*) were evaluated. The crude extract was analyzed in HPLC-DAD and fractions were also characterized using GC-MS and ESI-MS techniques. Results indicate phenolic compounds majority presence, including flavonol, anthraquinone and anthrones derivatives. Relative antimicrobial activities were detected against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida famata*, *Candida krusei* and *Candida tropicalis*. As well as, a weak anti-trypanosomatids activity against M2269 strain and BH46 strain of *Leishmania* sp. A relative antitumoral activity was also identified but in counterpoint were also observed a toxicity in fibroblast cells (L929). Phytochemically, results provide evidence that phenolic compounds in *S. rugosa* leaves might be the responsible for its antimicrobial activity and cell toxicity inferred in our research.
Key words: biological activity, phenolic compounds, phytochemical profile, *Senna rugosa*.

Resumo
Senna rugosa (Fabaceae) é uma espécie comum do território brasileiro, especialmente no Cerrado. É amplamente utilizado na medicina popular, embora ainda não tenha sido investigada quanto aos seus fitoconstituintes e atividades farmacológicas. Neste estudo, foram realizadas as análises fitoquímicas preliminares do extrato etanólico de folhas de *S. rugosa* e sua citotoxicidade, atividade antitumoral e antimicrobiana (antibacteriana, antifúngica, anti-*Trypanosoma* e anti-*Leishmania*). O extrato bruto foi analisado em HPLC-DAD e as frações foram caracterizadas usando técnicas de GC-MS e ESI-MS. Os resultados indicam a presença majoritária de compostos fenólicos, incluindo flavonóis, antraquinonas e derivados de antronas. Atividades antimicrobianas foram detectadas contra as espécies *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida famata*, *Candida krusei* e *Candida tropicalis*. Além disso, uma fraca atividade anti-tripanossomatídeos contra as cepas M2269 e BH46 de *Leishmania* sp. Uma atividade antitumoral foi identificada, porém também foi observada uma toxicidade sobre células de fibroblastos (L929). Fitoquimicamente, os resultados fornecem evidências de que compostos fenólicos nas folhas de *S. rugosa* podem ser os responsáveis pela sua atividade antimicrobiana e pela toxicidade celular observada em nosso estudo.
Palavras-chave: atividade biológica, compostos fenólicos, perfil fitoquímico, *Senna rugosa*.

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Introduction

Plants have an important role in the treatment of diseases and remain as the core of the traditional medicine systems in different countries (Newman & Cragg 2016). Brazil is a huge source of potentially useful plants in the medical standpoint. The genus *Senna* (Fabaceae) is widespread in Brazilian ecosystems, with some greatly appreciated species and widely used as ornamental plants for the beauty of its flowers. *Senna rugosa* (G. Don) Irwin & Barneby (1982: 35) is a medium-sized shrub that reaches up to 1.6 meters height (Dantas & Silva 2013). It can be easily identified by the massive amount of characteristic bright yellow flowers during the winter. It occurs in all regions of the Brazilian territory (BFG 2018) and is known for its therapeutic properties. In the popular medicine, *S. rugosa* has been reported as an anthelmintic remedy and a snakebites antidote (Rodrigues & Carvalho 2001). Like other species belonging to the genus, it is quite possible that there are many other activities yet to be associated with *S. rugosa*. The genus *Senna* is known to produce various classes of aromatic compounds, *e.g.*, quinones (Alemayehu *et al.* 1989), anthraquinones (Abegaz *et al.* 1994), naphthopyrones (Barbosa *et al.* 2004), triterpenoids (Li *et al*. 2012) and flavonoids (Baez *et al.* 1999). Natural anthraquinones are responsible for the cathartic effect commonly associated with species of the genus *Senna* (Dave & Ledwani 2012). However, *S. rugosa* was not adequately investigated as to its phytoconstituents and pharmacological activities.

Tropical diseases and bacterial infections are a major medical problem in global public health since different types of pathogens are encountered widespread in several countries. The current treatment of several of these diseases has large limitations. For instance, Leishmaniasis and Chagas disease are between the most important tropical neglected diseases affecting millions of people worldwide (WHO 2017, 2018a; Sundar & Chakravarty 2015). Unfortunately, the conventional therapies for this both diseases are toxic, has registry of resistance and low efficacy in some clinical forms, for example to chronic phase of Chagas disease and to diffuse cutaneous leishmaniasis (Griensven *et al.* 2016; Hendrickx *et al.* 2018; Kratz 2019).

Additionally, bacterial and fungal resistance to antimicrobial agents is too common, mainly in nosocomial and immunosuppressed patients, being responsible for significant morbidity, mortality and the increase in the treatment cost (Oliveira & Silva 2008; Silva 2010; Nakamura *et al.* 2013). Cancer represents another important and complex disease that affects populations worldwide (WHO 2018b). The treatment is performed by surgery, radiotherapy, chemotherapy or their combination. In general, chemotherapy is the most used alternative, but there have been limitations such as development of drug resistance, serious toxic effects and low efficacy in certain clinical forms (Luisi *et al.* 2006). Consequently, the discovery and development of new chemotherapeutic agents more effective and less toxic for these pathologies has received a great attention nowadays (Kunz & Brook 2010; Casellas 2011; Ventola 2015).

Use of plant extract and other natural compounds might represent an attractive option for discovery of new alternative therapeutic. Several studies have been conducted by different team and confirm the pharmacological potential the of plant-derived products as new drugs (Almagboul *et al.* 1985; Artizzu *et al.* 1995; Bhalodia & Shukla 2011; Thavamani *et al.* 2014). In this context, this study aim was to evaluate the phytochemical profile and its cytotoxicity and antiparasitic and antitumoral activities of the *Senna rugosa* ethanolic leaf extract. Furthermore, the crude extract was analyzed in High Performance Liquid Chromatography-Diode Array Detector Method (HPLC-DAD) and the initial characterization of the fractions by Gas Chromatography-Mass Spectrometry (GC-MS) and Electrospray Ionization Mass Spectrometry (ESI-MS) methods were also performed.

Materials and Methods

Chemicals and reagents

All the chemicals used were analytical grade reagents. Mercury (II) chloride, bismuth carbonate, iodine, metallic magnesium, cupric sulphate, sodium citrate, anhydrous sodium carbonate, sodium hydroxide, ammonium hydroxide, gelatin, sodium hydroxide, ferric chloride, lead acetate, rutin, emodin, aloe-emodin and Folin - Ciocalteu's phenol reagent were provided by Sigma-*Aldrich*® (St Louis, MO, USA). Concentrated hydrochloric acid, sulfuric acid and gallic acid were purchased by Dinâmica® (Diadema, São Paulo, Brazil). Silica gel 60G*70-230 mesh* and Aluminum TLC Plates SIL G/UV_{254} were acquired from Macherey-Nagel® (GmbH & Co.KG, Düren, Germany). Glacial acetic acid, potassium iodide, *n*-Hexane, ethyl

acetate, ethanol (96 ºGL) and methanol were provided by Synth® (Diadema, São Paulo, Brazil). Acetonitrile was provided by Panreac® (Castellar del Vallès, Barcelona, Spain).

Plant material and sample preparation

Senna rugosa species was identified and authenticated by the Ph.D. Carlos Victor Mendonça Filho, of the Department of Botany, UFVJM, Brazil. The leaves of *S. rugosa* were harvested at the University Campus of Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM), Campus JK, Alto do Jacuba, Diamantina, Minas Gerais, Brazil on 07.IV.2015 in the Cerrado field with appropriate Brazilian authorization to access the genetic heritage (nº 010832/2014-9). The voucher specimen (HDJF 4236) was deposited in this institution herbarium. Leaves of *S. rugosa* were dried in an oven with air circulation (Solab® SL102, São Paulo, Brazil) for 30 days at 39 ºC. After dried, the leaves were ground to powder using a knife mill (Solab® SL32, São Paulo, Brazil). The powder (100 g) was added to 1 L of solvent (ethanol 96 ºGL) and macerated for 7 days and this process was repeated 3 times. Thereafter the mixture was filtered, and the solvent was evaporated (39 ºC) using a rotative evaporator (Fisatom® 804, São Paulo, Brazil). The authorization of access to genetic resources (Process Registration Number: AACD7CF) was obtained from National System for Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN).

Fractionation

Ethanolic extract of *Senna rugosa* leaves was fractionated with *n*-hexane: ethyl acetate with an increasing polarity gradient, starting with 100% *n*-hexane up to 100% ethyl acetate. Subsequently, the eluent was exchanged to ethyl acetate: methanol increasing polarity gradient, starting with 100% ethyl acetate up to 100% methanol. A 1 g sample of the ethanolic extract was fractionated in eight fractions of *n*-hexane: ethyl acetate with the following ratios (v/v): 1:0 (1st and 2nd fraction), 95:05 (3rd fraction), 9:1 (4th and 5th fraction), 85:15 (6th fraction), 65:35 (7th fraction), 0:1 (8th fraction), using a silica gel 60 G packed chromatography column. From all mixtures of ethyl acetate: methanol, only the methanol 100% was able to elute the remaining constituents of the column (9th fraction). Following elution, the resultant fractions collected in test tubes were qualitatively evaluated using the thin-layer chromatography (TLC) performed on silica gel GF_{256}.

Plant samples phytochemical analysis

Phytochemical analysis was performed according to Simões *et al.* (2007). The specific chemical reactions assays were carried out on the crude extract and on the fractions samples in order to determine secondary metabolites presence such as tannins, phenols, steroids, triterpenes, saponins, flavonoids, alkaloids and anthraquinones.

Total phenolic content

Total Phenolic Content (TPC) was determined by the Folin-Ciocalteau method (Singleton *et al.* 1965) with some modifications. *Senna rugosa* crude leaf extract and the eighth and the ninth fraction were analyzed. Diluted samples (255 µl of 300 µg/mL extract solution) were mixed with 15 µl of Folin-Ciocalteu reagent (1:10 v/v diluted in water) and incubated for 3 min. It was added 30 µl sodium carbonate (1.0 N) and the mixture was incubated for 2 h at room temperature. The absorbance was measured at 765 nm (SpectraMax Paradigm Multi-Mode Microplate Reader UV/ Visible Spectrophotometer - Molecular Devices® Sunnyvale, CA, USA) and TPC was estimated based on a Gallic acid standard curve and expressed as milligrams of Gallic acid equivalents (GAE) g^{-1}. The result was reported as a mean value and standard deviation for three distinct assays.

HPLC-DAD analysis

Chromatographic analysis of crude extract was carried out using a Phenomenex® LUNA-C18(2) column (4.6 mm × 150 mm, 3 µm) (Shimadzu®, Kyoto, Japan) LC system consisting of quaternary pump LC-20AT, degasser DGU-20A5, the column oven CTO-20A, autosampler SIL-20ACHT with DAD detector SPD-M20A. The detection wavelength was set at 270 nm. The flow rate and injection volumes were 0.8 mL/ min and 10 µL, respectively. The mobile phase was A-ultrapure water (Direct-Q®/Millipore®) and B-acetonitrile (Panreac®, Castellar del Vallès, Barcelona, Spain), using gradient elution: 0–5 min, 10% B; 5–45 min, 10–100% B; 45–50 min, 100% B; 50–52 min, 100–10% B; 52–60 min, 10% B. The mobile phase was filtered through a 0.2 µm GHP membrane disc filter (47 mm diameter, Pall Corporation® Nova York, USA) and then denaturized ultrasonically prior to use. The column oven was set at 35 ºC.

Gas chromatography-mass spectrometry (GC-MS) analysis

Apolar fractions were injected into the GC (Perkin Elmer CLARUS 680 GC (Waltham®, MA, USA) with helium as carrier gas and a GC coated column with 5% diphenyl 95% dimethyl polysiloxane, 0.25 mm × 30 m (ELITE-5) and coupled to a spectrometer CLARUS 600T (Waltham®, MA, USA). The conditions were as follows: temperature programming from 60–240 °C (rate 3/min) holding at 240 °C for 15 min. The injection temperature was 220 °C and injection mode was split (ratio 50). The total flow was automatically adjusted, and the column flow was 0.6 mL/min. The temperature source was 190 °C and solvent cut time was 2 min. For the mass spectrometer in positive mode (EI^+), the start time was 2 min, end time was 75 min, scan time was 0.2 s, and start *m/z* ratio was 50 while end *m/z* ratio was 400 (Adams 2007). The mass spectrum was also equipped with a computer mass spectra data bank NIST v1.0.2.2.

Electrospray ionization mass spectrometry (ESI-MS) analysis

As no chromatographic separation was performed in the HPLC, the chemical profile of the fractions rich in acidic compounds (8^{th} e 9^{th}) was achieved by direct infusion in the ESI-MS (Shimadzu® LCMS-2020, Kyoto, Japan) set to negative mode. The identities of the main peaks in the phenolic fraction were suggested based on information reported in references (Gill & Morgan 2001; Bastos *et al.* 2007; Sang *et al.* 2007; Falcão *et al.* 2010; Navarre *et al.* 2011; Hamed *et al.* 2014; Spindola *et al.* 2014; Schmeda-Hirschmann *et al.* 2015; El Sayed *et al.* 2016).

Biological assays

Antibacterial activity assay

Four different species of bacteria were used from American Type Culture Collection (*ATCC-Rockville® MD, USA*): *Staphylococcus aureus* (ATCC 29313), *Salmonella typhimurium* (ATCC 14028), *Klebsiella oxytoca* (ATCC 49131) and *Pseudomonas aeruginosa* (ATCC 27853). The microdilution assay was carried by the guidelines of the National Committee for Clinical and Laboratory Standards Institute (CLSI 2012) using Mueller-Hinton medium (Himedia® Mumbai, India) to determine the minimal inhibitory concentration (MIC). All assays were performed in triplicate. Serial dilutions of plants extracts were prepared in sterile polystyrene 96-wells to range 1,000 to 125 µg/mL dissolved in dimethyl sulfoxide 0.125% (DMSO - Sigma-Aldrich® Sto Louis, MO, USA). The final concentration of bacterial suspension was adjusted to 5×10^5 colony-forming unit/mL in supplemented MH broth in the final volume of 100 µL. Media was used without the addition of extract or solvent for a growth and sterility control. As a control for the toxicity of the solvent, a culture was incubated with DMSO in the same concentration used in extract preparation. Conventional antibiotic chloramphenicol (Sigma-Aldrich® Sto Louis, MO, USA) was used as standard drug and after plates preparation and 24 h of incubation at 37 °C, 30 µl of resazurin solution 0.01% (Sigma-Aldrich® Sto Louis, MO, USA) was added to each well, incubated for 4 h at 37 °C, and assessed for color development. A change from blue to pink indicates the resazurin reduction and therefore bacterial growth. The MIC was defined as the lowest drug concentration that completely inhibits the growth of the organism in microdilution wells where no color change occurred.

The nature of the activity was also determined by adding 10 µL aliquots of all preparations which did not show visible growth during microdilution assay (MIC) in 10 mL of saline buffer 0.09%. After homogenization, 100 µL this preparation was spread in BHI agar medium plates. These plates were incubated at 37 °C for 24 h. All controls were also evaluated in this assay. For the absence of bacterial growth, the extracts were considered with bactericidal activity and the presence of growth were considered with bacteriostatic activity.

Antifungal activity assay

Minimum inhibitory concentration (MIC) was determined by broth microdilution method on according to Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI 2002a, b). All tests were conducted in triplicate in three different experiments and involved reference strains from the ATCC (Rockville® MD, USA): *Candida albicans* (ATCC 90028); *Candida famata* (ATCC 62894); *Candida krusei* (ATCC 34135); *Candida tropicalis* (ATCC 28707); *Aspergillus niger* (ATCC 10535) and *Penicillium expansum* (ATCC 1117). *Candida* species were maintained on Sabouraud dextrose agar (Himedia®, Mumbai, India) and incubated at 37 ºC for 48 h (CLSI 2002a). Filamentous fungi were harvested on solid YPD medium (Himedia®, Mumbai, India)

at 28 ºC for seven days (CLSI 2002b). Inoculum suspensions were prepared by spectrophotometric method corresponding to approximately 5.0×10^2 to 2.5×10^3 CFU/mL and 0.4×10^4 to 5.0×10^4 CFU/mL respectively. Yeast cells in the exponential phase were collected aseptically with a sterile loop and resuspended in a tube containing 5 mL of sterile saline. The suspensions were diluted in Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich® Sto Louis, MO, USA) containing L-glutamine and buffered to pH 7 with 0.165 M Morpholino Propanesulfonic Acid (MOPS - Sigma-Aldrich® Sto Louis, MO, USA). The extract was dissolved in DMSO (Sigma-Aldrich® Sto Louis, MO, USA) and subsequently, serial dilutions were prepared using RPMI as the diluent and tested at concentrations ranging of 1,000 to 125 µg/mL. For a growth and sterility control, media was used without the addition of extract or solvent. As a control for the toxicity of the solvent, a culture was incubated with DMSO. Amphotericin B 4 µg/mL (Sigma-Aldrich® Sto Louis, MO, USA) was utilized as the positive antifungal control. The inoculum of each fungal strain was added, and the plates were incubated at 37 ºC for 48 h for *Candida* species and 28 ºC for 72 h for filamentous fungi. Each test was performed in triplicate. The MIC was defined as the lowest drug concentration that prevented this color change.

Anti-trypanosomatids activity assays

Leishmania (Leishmania) amazonensis (MHOM/BR/73/M2269) and *Leishmania (Leishmania) infantum* (MHOM/BR/1070/BH46) was maintained at 26 ºC in Liver Infusion Tryptose medium (LIT - Camargo 1964), supplemented with 10% fetal bovine serum (Gibco Invitrogen® Grand Island, NY, USA) and the promastigotes forms were used for all experiments. The anti-leishmanial activity against promastigotes was determined in flat-bottomed 96-well plastic tissue-cultured plates. Promastigotes were counted in a Neubauer hemocytometer and for the assay 1×10^7 parasites/mL were seeded with different ethanolic extracts in the concentration range of 1,000 to 125 µg/mL. Commercial drug amphotericin B (5µg/mL - Sigma® Sto Louis, MO, USA) was used as the standard drug and positive control. The following controls were also used DMSO and Ethanol as solvents controls and pure culture as viability control. The plates were incubated at 26 ºC for 72 h and the viability of parasites was assayed colorimetrically by the mitochondrial oxidation of MTT (3-[4,5-dimethylthiazol-2-y1]-2,5diphenyl-tetrazolium bromide) as described by Mosmann (1983). The optical density was detected at 560 nm with a spectrophotometer detection platform (Spectra Max® Paradigm - Molecular Devices, Sunnyvale, CA, USA) and correlated with the number of living promastigotes. Control wells without any sample were established as 100% viable. Inhibitory concentration 50 (IC_{50}) was determined by the logarithmic regression analysis method (Sebaugh 2011).

Two *Trypanosoma cruzi* strains were used for evaluating the anti-*Trypanosoma* activity: Y and Colombian strains which are considered prototypes of partial susceptibility and resistant to benznidazole respectively (Filardi & Brener 1987). Epimastigotes were harvested axenically in LIT at 28 ºC, being supplemented with 10% fetal bovine serum (Gibco Invitrogen® Grand Island, NY, USA) and used in all experiments in the stationary phase. The assays were performed in the same conditions described above for anti-leishmanial activity, but Benznidazole (BZ) was used as standard drug in 75 µg/mL concentration (Muelas-Serrano *et al.* 2000).

Antitumoral activity assay

Human breast cancer cell lines MDA-MB-231 were obtained from ATCC (LGC Promochem® Rockville, MD, USA) and used to determine the antitumoral activity of the crude extract. The MDA-MB-231 were maintained in Dulbecco's modified Eagle medium (DMEM - Sigma-Aldrich® Sto Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics (100 mg/mL of streptomycin and 100 unit/mL of G penicillin, Sigma-Aldrich® St. Louis, USA). Cells were maintained in the exponential growth phase at 37 ºC in a humidified incubator with an atmosphere of 5% CO_2 and submitted periodical peal. The cells were plated 1.5×10^5 cells/well in a 96-well flat-bottom plates and allowed to adhere for 24 h. Paclitaxel 6 ng/mL (Quiral Química®, Juiz de Fora, Brazil) was used as a standard drug (PXT). Cells without a test compound were used as a viability control, DMSO used as a solvent toxicity control while the condition with only medium was used as blank. After incubation for 72 h with the crude extract in the concentration range of 1,000 to 7.8 µg/mL, the cell viability was measured by the MTT assay as previously described by Mosmann (1983). The relative growth rate was calculated to evaluate the extract antitumoral activity. Inhibitory

concentration 50 (IC_{50}) was determined by the method of logarithmic regression analysis of data obtained. All analysis was performed in triplicate, and the average data were reported as the results.

Cytotoxicity cellular assay

Mouse fibroblast cells (L929) were maintained at RPMI (Sigma-Aldrich® Sto Louis, MO, USA) using the same conditions of supplementation above. For cell viability assay, 1×10^5 cells/well were seeded into 96-well plates followed by the addition of the *Senna rugosa* ethanolic leaf extract tested in the concentration ranging of 1,000 to 7.8 µg/mL prepared in cell culture media. Cell viability was measured by MTT assay as previously described by Mosmann (1983) and the 50% cytotoxic concentration (CC_{50}) was calculated. Cells without a test compound were used as a viability control, DMSO was used as a solvent toxicity control and only the medium served as the blank. The cadmium chloride ($CdCl_2$) (200 nM - Quiral Química®, São Pedro, Juiz de Fora, Brazil) was used as death control.

Statistical analysis

The statistical analyses were performed using Graph Pad Prism 3.0 software. Results were expressed as the mean ± standard deviation of triplicate samples from three independent assays and were analyzed using the analysis of variance (ANOVA one way) with Bonferroni multiple comparison adjustment for parametric data. Significant differences from the control group were determined by Dunnett's test for non-parametric data, with $p < 0.05$ as the significance criterion. The EC_{50} values were calculated using sigmoid dose-response curves in Graph Pad Prism.

Results and Discussion

Preliminary phytochemical analysis and thin-layer chromatography of the plant extract and fractions

After ethanolic extract preparation 5.7349 g of crude leaf extract of *Senna rugosa* were obtained corresponding to 5.73% of yield. Preliminary phytochemical analysis results showed that *S. rugosa* leaves contain flavonoids, tannins, triterpenes and anthraquinones. TLC analysis showed that 5th, 6th and 7th fractions are mixtures of pigments (chlorophylls and carotenoids). Using *n*-hexane as eluent in TLC;1st, 2nd, 3rd and 4th fractions have low retention factor (Rf) values: 0.77; 0.72; 0.7 and 0.54 respectively, suggesting the presence of apolar compounds such as leaf waxes, fatty alcohols and other similar organic compounds. Rf high values for the dark-brownish residues obtained (8th and 9th fractions) and immediate solubility in alkaline solutions revealing an acid character of these constituents being consistent with phenolic compounds.

Anthraquinone species have already been described in roots of *S. rugosa* (Barbosa *et al.* 2004). Anthraquinones are secondary metabolites very common in Fabaceae family to which this species belongs (Bruneton 2001; Sakulpanich & Gritsanapan 2008; Sahu *et al.* 2014), confirming our data achieved by phytochemicals tests and ESI -MS results.

Triterpenes and steroids were also found in ethanolic extracts of the leaves and of the seeds of *S. rugosa* (Farias *et al.* 2010). Other studies also identified the presence of flavonoids in the leaves of *S. rugosa* (Eberhardt 2012). In the present study, the result of phytochemical screening of the ethanolic leaf extract of *S. rugosa* was also identified tannins. Moreover, Farias *et al.* (2010) failed to detect such a metabolite class in the *S. rugosa* aqueous seed extract. However, several studies have documented the possibility of identifying different chemical compositions in the extract from the same plant species (Azevedo *et al.* 2014), since a yield or result of extraction depends on the solvent with varying polarity, the composition of the sample, pH, extraction time and temperature. In addition, plant parts used on different research are not always the same and what could also justify the divergence of results is due to the genetic and the ontogeny variations among individuals or in environmental conditions that may alter the metabolism.

Total phenolic content

The content of phenolic compounds in *S. rugosa* ethanolic leaf extract and in the 8th and 9th fractions with the acid character was determined through a linear Gallic acid standard curve ($y = 0.003968x + 0.486683$; $r^2 = 0.9924$). The total phenolic content of the 8th fraction was 325.87 ± 12 mg GAE/g and of the 9th fraction was 152.4 ± 5.94 mg GAE/g (Tab. 1). Phenolic compounds detected by the Folin-Ciocalteau method can be classified into simple phenols and polyphenols. Simple phenols include phenolic acids, coumarins among other molecules. Nevertheless, polyphenols cover the flavonoids, stilbenes, lignans and tannins (Balasundram *et al.* 2006; Tsao 2010). Anthraquinones can be simple phenols or

Table 1 – Total phenolic content of *Senna rugosa* leaf ethanolic extract.

Sample	Phenolic compounds (mg GAE.g^{-1} of extract)
Ethanolic extract	43.87 ± 2.69
8º fraction	325.8 ± 12
9º fraction	152.4 ± 5.94

GAE = Gallic Acid Equivalent

polyphenols, depending on its molecular structure and is considered one of the major plant phenolic pigments (Mohammed 2016). Simple phenols and the polyphenols present in plants are subjects of increasing scientific interest due to their potential biological action, particularly in pharmacological terms and possible beneficial effects on human health. Polyphenols are secondary metabolites generally involved in defense against aggression by pathogens or ultraviolet radiation (Beckman 2000). Additionally, polyphenols are some of the principal compounds related to the benefits of a diet rich in different plant parts, tea and wine, due to their antioxidant properties. The values obtained in Folin-Ciocalteau method achieved in this work are in accordance with three classes of metabolites detected in the preliminary phytochemical analysis (anthraquinones, flavonoids and tannins).

Analysis of crude extract by HPLC-DAD

The HPLC-DAD results revealed the presence of a major peak in the crude extract, with time retention at 17.22 min (Fig. 1). Aiming to identify the major secondary metabolite of this extract, some

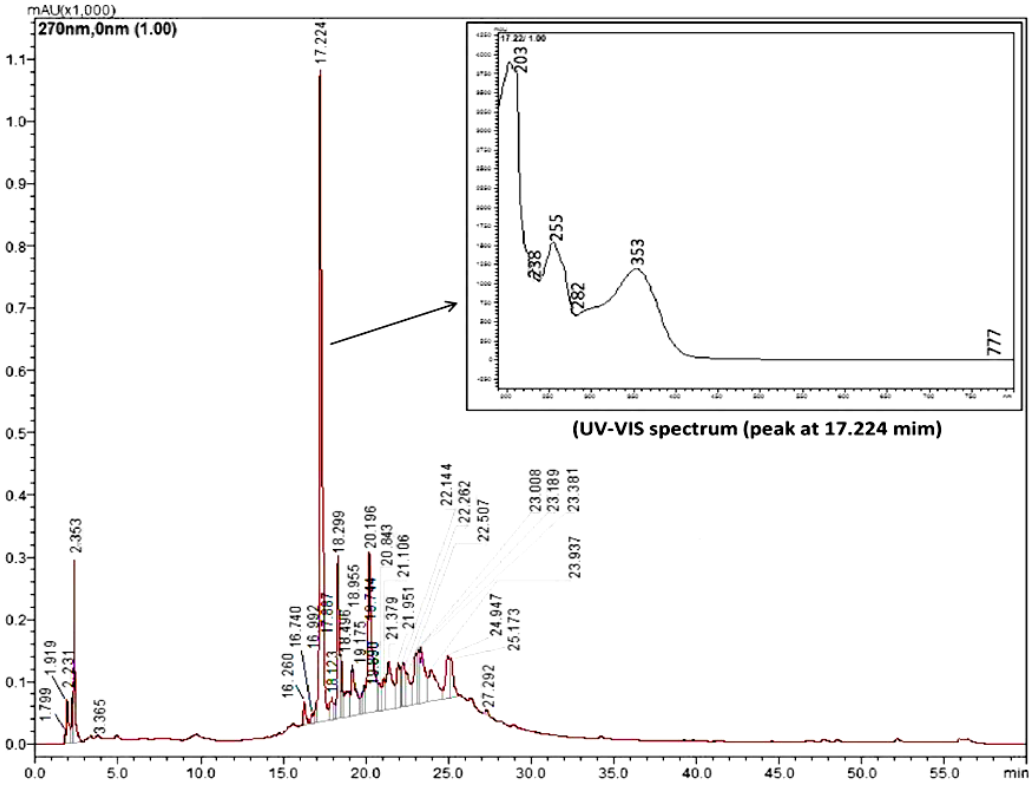

Figure 1 – HPLC-DAD chromatogram at 270 nm of ethanolic extract of *Senna rugosa* and UV-Vis spectrum of the major component identified in HPLC method.

standards were chosen for elution in HPLC-DAD under the same conditions as those in the crude extract. Considering the results of studies of other researchers (Barbosa *et al.* 2004) and the common anthraquinones and flavonoids in this genus (Baez *et al.* 1999; Barbosa *et al.* 2004), the standards rutin, emodin and aloe-emodin (Sigma-Aldrich® Sto Louis, MO, USA) had their retention times and UV-Vis spectra compared to the crude extract chromatogram. The analysis of the results of the standards indicates that the major peak is the rutin (flavonol glycoside). The comparison of the data did not reveal the presence of emodin and aloe-emodin in the crude extract. This result may be evidence of a low concentration of anthraquinones and could be evidenced by more sensitive techniques.

Analysis of fractions by GC-MS and ESI-MS

The GC-MS analysis of the ethanolic extract led to the identification of 8 compounds including leaf waxes (octacosane, decane and heptacosane) fatty alcohol (phytol), aromatic hydrocarbons (ethylbenzene, m-xylene and p-xylene) (Fig. 2) and 1-Methylpentyl Hydroperoxide (Tab. 2).

Acidic constituents' analysis in the ESI-MS (negative mode) showed the presence of peaks traditionally associated with phenolic compounds (Tab. 3; Figs. 3; 4). However, the full identification of compounds requires more detailed studies. The peak at 416/417 confirms the positive results for anthraquinones (Barbaloin/isobarbaloin) given by the preliminary phytochemical analysis (Tab. 3).

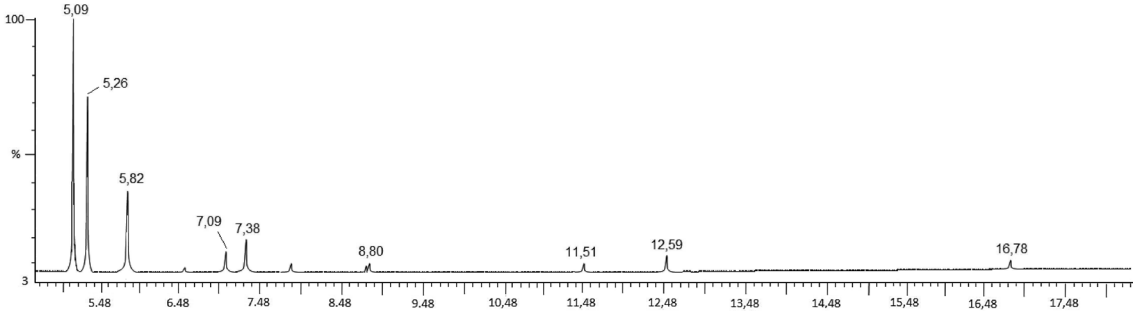

Figure 2 – Chromatogram with most prominent peaks of ethylbenzene, *m*-xylene and *p*-xylene in GC-MS chromatogram of the 2nd fraction of ethanolic extract of *Senna rugosa* leaf. For try to relate the phytoconstituents to the retention time, please see Table 3 (sample N° 2).

Table 2 – Identified peaks in fractions by GC-MS of *Senna rugosa* leaf ethanolic extract.

Sample N°	Compound	Retention Time (min)	Molecular weight	Major peaks
1.	Octacosane	32.98	394.76	57, 43, 71, 85
2.	Ethylbenzene	5.09	106.16	91, 106
	m-Xylene	5.26	106.16	91, 106
	p-Xylene	5.82	106.16	91, 106
	1-Methylpentyl Hydroperoxide	7.38	118.17	43, 41
	Decane	11.51	142.29	43, 57
3.	Heptacosane	33.89	380.73	57, 43, 71, 85
4.	Phytol	31.11	296.54	71, 43, 57, 81

Table 3 – Analysis of 8th and 9th fractions of *Senna rugosa* leaves extract by ESI-MS of low resolution (Negative Mode).

Fraction	$[M-H]^-$	M.W	Suggested Compounds
8th	302	-	Unknown
	417/416	418.39	Barbaloin / isobarbaloin
	543	544.14 544.50	Proanthocyanidin (E)afzelechin-(E)afzelechin or Diferuloylquinic acid (isomers)
	657	658.56	Dihydromyricetin diglucoside
	769	770.78	Proanthocyanidin trimer (prodistenidin C)
	883	884.7	P2 analogs (EGCG* degradation)
	1009/1010	-	Proanthocyanidin oligomers
9th	249	250.29	Caffeoyl putrescine
	255	256.25	Emodin anthrone / Aloe-emodin anthrone
	379	-	Unknown
	403	404.41	Pinocembrin derivative
	515	516.45	Dicaffeoylquinic acid isomers

* = epigallocatechin-3-gallate

Moreover, Barbaloin/isobarbaloin and Emodin anthrone/Aloe-emodin anthrone, are according to the class of substances common to the genus. Although anthraquinone derivatives have not been shown in HPLC-DAD, this fact indicates an increase in the concentrations of these compounds provided by the fractioning step, associated with a more sensitive characterization method ESI-MS, allowing the identification in this step. The evidence of anthraquinones generates the possibility of future research on the cathartic action of fractions of the *Senna rugosa* leaf extract. Otherwise, the identification of rutin in HPLC-DAD and non-detection in ESI-MS indicate the loss of the compound throughout the fractionation step.

Biological results

The leaf extract demonstrated relative antimicrobial activity against *Staphylococcus aureus* (Gram-positive species) with MIC of 500 µg/mL and *Pseudomonas aeruginosa* (Gram-negative species) with MIC of 250 µg/mL. The polyphenols compounds present in various plant species are well documented to have microbial activities against many pathogenic bacteria (Scalbert 1991; Cowan 1999). The total phenolic compound contents in *Senna rugosa* ethanolic leaf extract and the rich phenolic compounds content in the 8th and 9th fractions (Tab. 1) provides some inkling for the action of polyphenols on antimicrobial activity demonstrated.

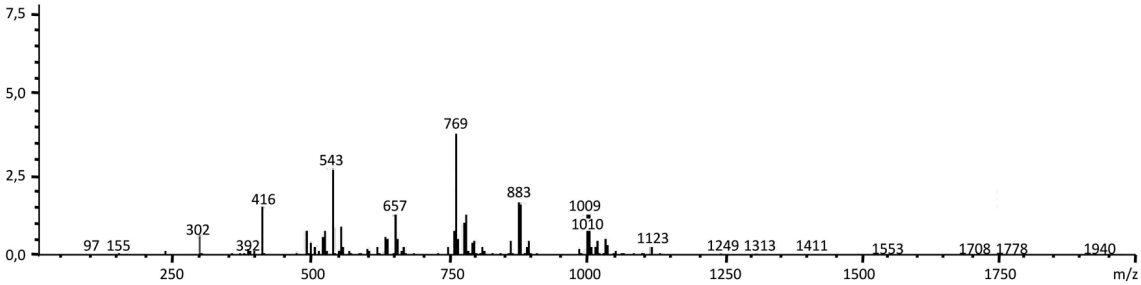

Figure 3 – Mass spectrum (ESI-MS) of 8th fraction of *Senna rugosa* leaf extract in negative mode.

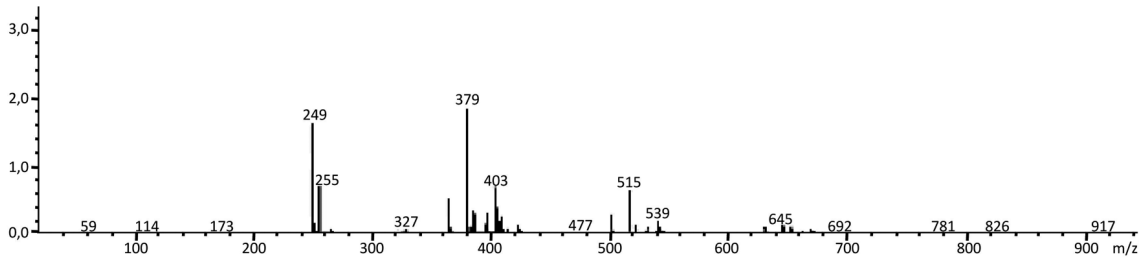

Figure 4 – Mass spectrum (ESI-MS) of 9th fraction of *Senna rugosa* leaf extract in negative mode.

Activity against *S. aureus* was also observed by Ederhardt (2012) which demonstrated a bacteriostatic effect corroborating the data obtained in our studies. Anthraquinones (Chrysophanol), flavonoids (kaempferol) and terpenoids (β-sitosterol) obtained from *Senna reticulata* (Willd.) H.S. Irwin & Barneby (1982: 35) were associated to antimicrobial activity (Kataoka *et al.* 2001; Singh & Singh 2003; Garcia-Sosa *et al.* 2006; Santos *et al.* 2008). All these secondary metabolite classes were also observed in the phytochemistry screening of leaf extract of *Senna rugosa.* The crude extract was tested against six pathogenic fungi and effective antifungal activities were observed against *C. famata* (CIM of 62.5 µg/mL) *C. krusei* (62.5 µg/mL) and *C. tropicalis* (CIM de 125 µg/mL), in all cases with fungicidal action. The antifungal activity has already been associated to other species belonged *Senna* genus, for example, *S. alata* (L.) Roxb. (1832: 349) (Sule *et al.* 2010) and *S. obtsufolia* (L.) H.S. Irwin & Barneby (1831: 252) (Doughari *et al.* 2008). Anthraquinones isolated from *Senna alata* showed activity against several fungi species (Wuthi-Udomlert *et al.* 2010) and this metabolic were also observed in the *Senna rugosa* ethanol leaf extract. Several species of the genus *Senna* were not also active against *Candida albicans* including leaf ethanolic extracts of *Senna podocarpa* (Guill. & Perr.) Lock (1988: 340) and *Senna tora* (L.) Roxb. (1832: 340) and *Senna spectalbilis* (DC.) H.S. Irwin & Barneby (1962: 600) (Chukeatirote *et al.* 2007; Ogundare 2009).

The trypanocidal assay not showed activity against neither *T. cruzi* strains as summarized in Figure 5. For anti-leishmanial activity was observed a weak effect of the extract on the concentration of 1,000 and 500 µg/mL for the strain M2269 and only of 1000 µg/mL for the strain BH46 (Fig. 6).

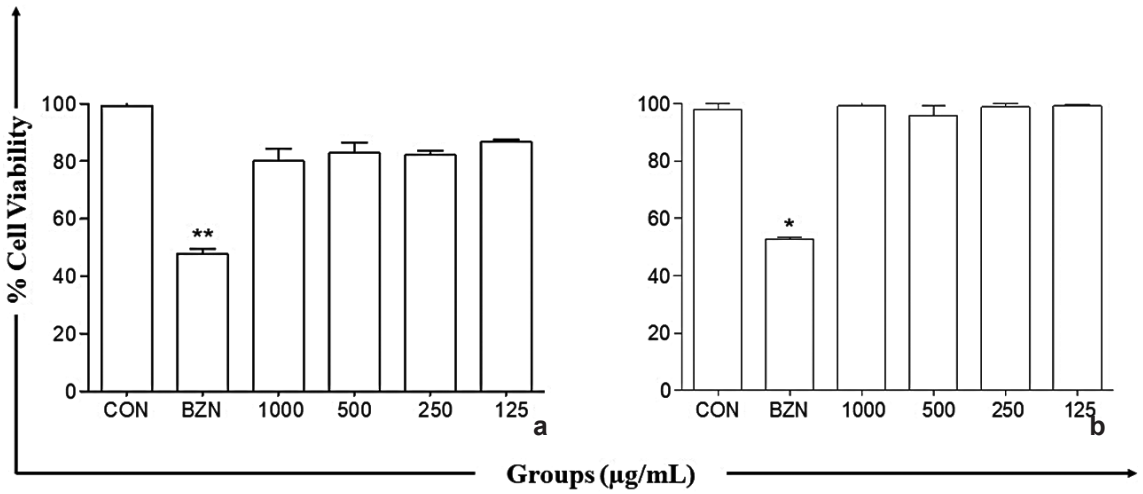

Figure 5 – a-b. Results obtained from *in vitro* assay of *Senna rugosa* leaf extract against epimastigotes form from *Trypanosoma cruzi – a.* Y strain; b. Colombian strain. The results were expressed as the average percentage of viability ± standard deviation of three assays. Assays were carried out in triplicate and compared with results obtained for the control of cell (CON). * $p < 0.05$ and ** $p < 0.01$. BZN- benznidazole was used as standard drug.

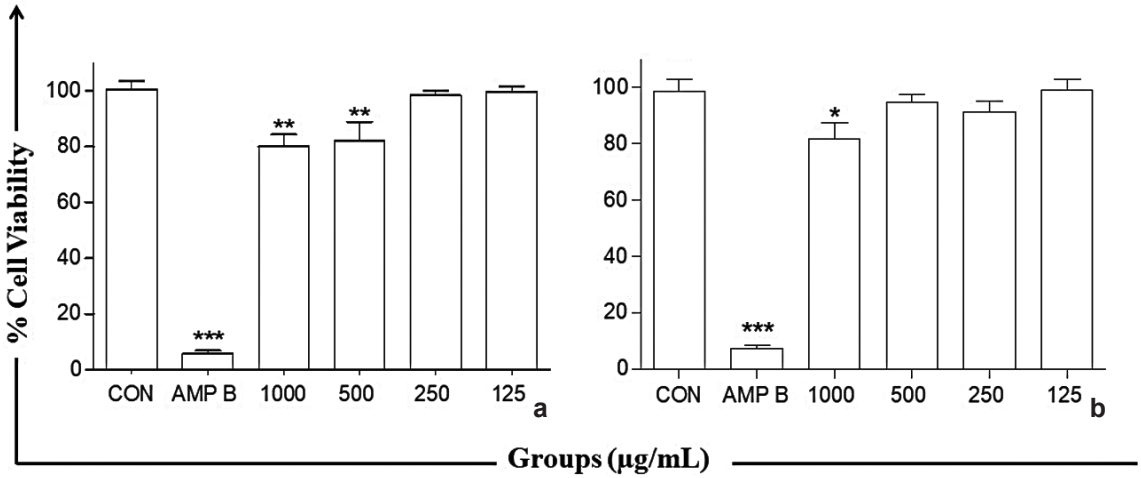

Figure 6 – a-b. Results obtained *in vitro* activity assay of *Senna rugosa* ethanolic leaf extract against promastigotes – a. from *Leishmania* (*Leishmania*) *amazonensis amazonensis* M2269; b. from *Leishmania* (*Leishmania*) *infantum* BH46. The results were expressed as the average percentage of cell viability ± standard deviation of three assays. Assays were carried out in triplicate and compared with results obtained for the control of cell (CON). * $p < 0.05$; **$p < 0.01$ and *** $p < 0.001$. AMP B-Amphotericin B was used as standard drug.

Antitumoral activity evaluation of *Senna rugosa* ethanolic leaf extract against MDA-MB-231 cells showed significant activity at concentrations 1,000–250 µg/mL (cell viability reduction ranging from 87.37% to 14.91% and IC_{50} = 607.9µg/mL) showed in the Figure 7. Given the efficacy relative to paclitaxel (100% efficiency and percentage of cell death 85.66%), was found greater effectiveness in the highest tested concentration corresponding to 111.38%. Potential antitumoral activity were observed in other species of *Senna* genus, for example *Senna alata* (Olarte *et al.* 2013); *Senna tora* (Zhao *et al.* 2013); *Senna italica* Mill. (1768: 8(2)) (Masoko *et al.* 2009), *Senna gardneri* (Benth.) H.S. Irwin & Barneby (1982: 192), *Senna splendida* (Vogel) H.S. Irwin & Barneby (1982: 190) (Silva *et al.* 2014) and *Senna occidentalis* (L.) Link (1829: 140) (Essien *et al.* 2011). Considering the antitumoral activity despite the good efficiency by *Senna rugosa* leaf extract the selectivity index (SI) was only 0.79. Because the *S. rugosa* extract showed high toxicity to the murine fibroblast cells (L929) with CC_{50} of 480.8 µg/mL and leading to viability reduction of 53.9 and 14.65% for the concentration of 1,000 and 31.25 µg/mL respectively (Fig. 8).

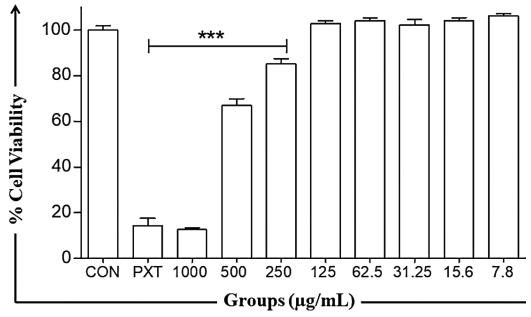

Figure 7 – Results obtained from *in vitro* assay for antitumoral activity assay of *Senna rugosa* ethanolic leaf extract against MDA-MB-231 cells. The results were expressed as the average percentage of viability ± standard deviation of three assays. Assays were carried out in triplicate and compared with results obtained for the control of cell (CON). *** $p < 0.001$. PXT = paclitaxel used as standard drug.

Toxicity was observed in other species belonged to *Senna* genus evaluated *in vivo* model (Yagi *et al.* 1998; Momin *et al.* 2012). *Senna occidentalis* was described as toxic species due to the presence of anthraquinone which causes the uncoupling of mitochondrial oxidative phosphorylation (Yadav *et al.* 2010). This secondary metabolic was also observed in the *S. rugosa* leaf. In the same way, flavonoids have been observed and associated with cell toxicity in other species (Levy & Carley 2012; Silva *et al.* 2014).

Conclusions

In this present study, we performed the chemical and biological profile of *Senna rugosa* ethanolic leaf extract. The data obtained in our

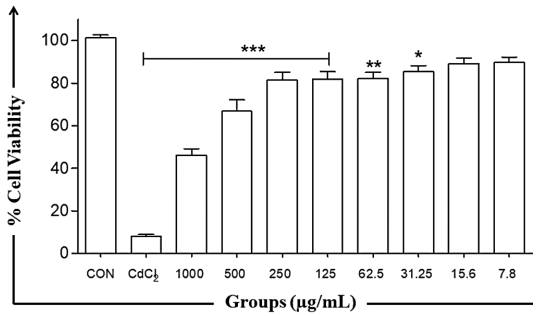

Figure 8 – Results obtained from *in vitro* cytotoxicity assay of *Senna rugosa* leaf extract against cellular viability of mouse fibroblast cells (L929). The results were expressed as the average percentage of viability ± standard deviation of three assays. Assays were carried out in triplicate and compared with results obtained for the control of cell (CON). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$. $CdCl_2$ = cadmium chloride used as cell death control.

research show that *S. rugosa* showed a chemical profile rich in phenolic compounds, such as rutin, anthraquinones and anthrones derivatives. And these compounds have shown diverse pharmacological activities justifying further studies, among them the cathartic function that is historically associated with the genus. For anti-trypanosomatids was observed only a weak against M2269 strain and BH46 strains of *Leishmania* sp. And although it has been observed a high antitumoral activity a high cytotoxicity in fibroblast cells (L929) was also observed, and the SI was very low (0.79). Considering the antimicrobial activity, were observed particularly promising activity against the species *Staphylococcus aureus* and *Pseudomonas aeruginosa* (bacteria species) and *Candida famata*, *Candida krusei* and *Candida tropicalis* (fungi species) with strong possibility that these activities could also be linked to the presence of phenolic compounds as observed for other species of *Senna* genus.

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